Black, V. L., Heesom, K., Whittington, F., Davis, S., Tasker, S., Adamantos, S. E., & Davidson, A. D. (2018). Assessment of the red blood cell proteome in a dog with unexplained hemolytic anemia. *Veterinary Clinical Pathology*, *47*(3), 377-385. https://doi.org/10.1111/vcp.12627

Assessment of the red blood cell proteome in an unexplained hemolytic anemia in a dog.

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Abstract

A 7-year old female neutered Jack Russell Terrier was presented to Langford Vets, University of Bristol with a chronic history of intermittent lethargy. Investigations and clinical course were compatible with hereditary hemolysis due to a red blood cell membrane defect. Proteomics was utilized to explore protein alterations in the presence of a hypothesized red blood cell membrane protein deficiency. The proteomic analysis revealed a down-regulation of the proteins band 3, alpha and beta adducin and alterations to the red blood cell proteome consistent with previous reports of changes accounted for by the presence of a reticulocytosis and ongoing hemolysis. The spectrum of protein alterations identified in the affected dog may be homologous to a band 3 protein deficiency secondary to hereditary spherocytosis as described in man.

Key words: dog, hemolysis, osmotic fragility, membrane, proteomics, red blood cell

Case presentation

Hemolysis as a result of hereditary hemolytic anemia has been described in people and domestic animals including the dog and cat[1-3]. Etiologies of hereditary hemolytic anemia can be categorized as enzymopathies, hemoglobinopathies, or red blood cell (RBC) membrane defects[2]. Clinical signs in affected patients are variable; some phenotypes are typically fatal in early life whereas others are reported to be detected as an incidental finding[3,4]. Despite a high index of suspicion, pinpointing the underlying defect can be challenging in human medicine[5]. In people diagnosed with hereditary spherocytosis where clinical features are more severe than the hypothesized phenotype, further investigations into the underlying etiology are recommended. Recent published guidelines recommend the use of SDS-PAGE

to explore alterations to the erythrocyte protein profiles, followed by sequence analysis of the membrane protein genes when the protein results do not explain the clinical outcome or mode of inheritance[6]. In tandem with these recommendations, high-throughput proteomic analysis has been applied to patients presenting with hereditary hemolysis and in some cases identified hypothesized protein deficiencies[5,7].

Red blood cells, aside from reticulocytes, are particularly attractive for proteomic analysis due to their lack of internal membrane bound organelles and nucleus, resulting in limited complexity. Proteomics has been applied to the study of RBCs since 2002 with particular emphasis on the assessment of protein defects in specific disease presentations, in changes to RBCs over time with storage, and in directing development of new drugs, for example anti-malarial drug therapy[8].

The following report describes to the authors' knowledge the first application of proteomics to further explore the cause and consequences of a presumed RBC membrane protein deficiency in a dog presenting with hereditary hemolysis.

A 7-year old female neutered Jack Russell Terrier was presented with a history of chronic intermittent lethargy. CBC (EDTA blood, Abbot Cell-dyn 3700) and biochemistry (serum, Komelab Prime 60i) were performed. Hemolysis was suspected based upon a persistent reticulocytosis, occasional spherocytes, and an acute drop in hematocrit during a 24 hour hospitalization period associated with lethargy, hemoglobinuria and stable plasma proteins (Table 1, Day 30 to Day 31).

Investigations were directed at the potential causes of hemolysis, specifically immune-mediated disorders, toxins, or infectious causes. Immune-mediated RBC destruction including immune-mediated hemolytic anemia and hemophagocytic syndrome were excluded based on history, clinical course and investigation findings including a negative direct antiglobulin test (rabbit anti-canine complement (C3b), IgG and IgM sera at 4 and 38°C, Langford Vets Diagnostic Laboratories, University of Bristol), and, thoracic and abdominal CT imaging, ultrasound guided hepatic and splenic fine-needle aspirates, and bone marrow aspirate cytology respectively. Findings revealed extramedullary hematopoiesis and hemosiderin within the liver and spleen and erythroid hyperplasia on bone marrow cytology, consistent with increased RBC turnover. Imaging did not reveal a metallic foreign body and evidence of ongoing compensated hemolysis had been documented over a two month period with no known exposure, therefore a toxin was considered unlikely. Infectious disease screening for *Babesia* spp., haemoplasma spp., *Ehrlichia* spp., *Anaplasma* spp., and *Leptospira* spp. was negative.

Hemoglobin-oxygen dissociation and osmotic fragility testing were performed to assess for a hereditary RBC defect. Samples from the affected case and the healthy control dog were shipped on ice and processed simultaneously for osmotic fragility testing and hemoglobin-oxygen dissociation curves at PennGenn (PennGenn Laboratories, University of Pennsylvania) as previously described.[1,9] The hemoglobin-oxygen dissociation curve was found to be comparable to the control and within the reference range (see Figure 1). Hemoglobinopathies and RBC enzyme deficiencies including pyruvate kinase and phosphofructokinase deficiencies result in a shift in the oxygen-dissociation curve[10-14], and thus were considered unlikely. In contrast, RBC membrane disorders are not anticipated to

result in a shift in the oxygen-dissociation curve and were therefore still considered a possibility in the affected case[15].

The patient had a markedly abnormal osmotic fragility (OF) test result (Figure 2, case 50% hemolysis at 67% saline dilution, control at 41% saline dilution), consistent with increased RBC fragility. The lysis curves for erythrocytes from the control and affected dog did not overlap, indicating the entire population of RBCs were more fragile[1]. The OF test evaluates the extent of hemolysis of RBCs at increasingly hypotonic solutions of saline. Hemolysis occurs due to a breakdown in osmotic regulation and loss of volume control, resulting in cell swelling and concomitant loss of surface until they reach a critical hemolytic volume after which lysis occurs[16]. RBCs can have an increased tendency to hemolyze during OF testing due to diminished active transport (e.g. pyruvate kinase deficiency), increased membrane permeability (e.g. spectrin deficiency)[17] and/or reduced RBC surface area (e.g. spherocytes)[16]. The results of the hemoglobin-oxygen dissociation and osmotic fragility testing, in combination, were supportive of a RBC membrane defect in the affected dog[18].

Scanning electron microscopy was performed to further evaluate the RBC structure (Figure 3) of the case and control dog. Blood samples for electron microscopic studies were anticoagulated with lithium-heparin and 80µl of this heparinized blood was mixed with 500µl of 2.5% glutaraldehyde (v/v) in 0.1M HEPES buffer at pH 6.8. Aliquots of samples were applied to glass slides and samples were dehydrated though a graded ethanol series before critical point drying. Samples were sputter-coated with 15 nm of silver and examined with a Jeol 6330 field emission scanning electron microscope. No obvious structural abnormalities were identified.

Based upon a strong suspicion of a hereditary RBC membrane protein deficiency, proteomic analysis of the RBC membrane proteins was performed using quantitative high-throughput mass spectrometry and the relative amounts of the proteins identified in the patient compared to those in a healthy age matched control dog determined.

Proteomic analysis

Red blood cell ghost preparation

Four samples from the control and affected dog were treated individually, pooled and analyzed (i.e. four per animal). RBC membrane ghosts were prepared from EDTA anticoagulated blood by washing 2.5ml of sample six times in 25ml phosphate buffered saline at pH 7.4 with aspiration and removal of the supernatant and buffy coat after centrifugation on a density gradient (600 x g for 5 min, Histopaque 1077; Sigma-Aldrich). The RBCs were then lysed by adding 10 volumes of ice-cold lysis buffer (20mM Tris-HCl, pH 7.6) and pelleted by centrifugation at 40,000 g for 30 min at 4°C. The supernatant and opaque buttons of residual leukocytes were removed using a pipette, leaving the translucent RBC membranes. The membranes were washed a minimum of four times in ice-cold lysis buffer, until the supernatant was clear of hemoglobin and the RBC ghosts were obtained. After the final wash the supernatant was again discarded and 200µl of lysis buffer added. The concentration of proteins in each sample was measured against a standard curve using the Qubit® Protein Assay and the Qubit 2.0 Fluorimeter (Invitrogen Eugene, Oregon, USA, Turner Biosystems). The samples were then stored at -80°C prior to proteomic analysis.

Tandem Mass Tag (TMT) labelling and cation exchange chromatography

Aliquots of 63µg of control (700µl) and case (300µl) protein extracts were digested with trypsin (2.5µg trypsin per 100µg protein; 37°C, overnight) and labelled with TMT 6plex reagents according to the manufacturer's protocol (Thermo Fisher Scientific, Loughborough, LE11 5RG, UK). After labelling, samples were combined and a 50µg aliquot of this pooled sample evaporated to dryness and resuspended in 50µl of Buffer A (10mM KH_2PO_4, 25%MeCN pH 3) prior to fractionation by strong cation exchange using an Ettan LC system (GE Healthcare). In brief, 48µl of sample was loaded onto a PolysulphoethylA column (100 x 2.1mm, 5µm, 200A; PolyLC Inc.) in buffer A and peptides eluted with an increasing gradient of buffer B (10mM KH_2PO_4, 25%MeCN 1M KCl pH 3) from 0-100% over 30 minutes. The resulting 15 fractions were evaporated to dryness, resuspended in 5% formic acid and then desalted using SepPak cartridges according to the manufacturer's instructions (Waters, Milford, Massachusetts, USA)). Eluate from the SepPak cartridge was again evaporated to dryness and resuspended in 40µl of 1% formic acid prior to analysis by nano-LC MSMS using an LTQ-Orbitrap Velos Mass Spectrometer with 15µl for each run.

Nano-LC Mass Spectrometry

Cation exchange fractions were further fractionated using an Ultimate 3000 nanoHPLC system in line with an LTQ-Orbitrap Velos mass spectrometer (Thermo Scientific). In brief, peptides in 1% (vol/vol) formic acid were injected onto an Acclaim PepMap C18 nano-trap column (Thermo Scientific). After washing with 0.5% (vol/vol) acetonitrile 0.1% (vol/vol) formic acid, peptides were resolved on a 250 mm × 75 µm Acclaim PepMap C18 reverse phase analytical column (Thermo Scientific) over a 150 min organic gradient, using 7 gradient segments (1-6% solvent B over 1min, 6-15% B over 58min, 15-32%B over 58min,

32-40%B over 5min, 40-90%B over 1min, held at 90%B for 6min and then reduced to 1%B over 1min.) with a flow rate of 300 nl min^{-1}. Solvent A was 0.1% formic acid and Solvent B was aqueous 80% acetonitrile in 0.1% formic acid. Peptides were positively ionized by nano-electrospray ionization at 2.0kV using a stainless steel emitter with an internal diameter of 30 μm (Thermo Scientific) and a capillary temperature of 250°C. Tandem mass spectra were acquired using an LTQ-Orbitrap Velos mass spectrometer controlled by Xcalibur 2.0 software (Thermo Scientific) and operated in data-dependent acquisition mode. The Orbitrap was set to analyze the survey scans at 60,000 resolution (at m/z 400) in the mass range m/z 300 to 1800 and the top ten multiply charged ions in each duty cycle selected for MS/MS fragmentation using higher-energy collisional dissociation (HCD) with normalized collision energy of 45%, activation time of 0.1 ms and at a resolution of 7500 within the Orbitrap. Charge state filtering, where unassigned precursor ions were not selected for fragmentation, and dynamic exclusion (repeat count, 1; repeat duration, 30s; exclusion list size, 500) were used.

The raw data files were processed and quantified using Proteome Discoverer software v1.2 (Thermo Scientific) and searched against the UniProt *Canis familiaris* database [9615] (database downloaded 31-12-2014; 28492 entries) using the SEQUEST algorithm. Peptide precursor mass tolerance was set at 10ppm, and MS/MS tolerance was set at 20mmu. Search criteria included oxidation of methionine (+15.9949) as a variable modification and carbamidomethylation of cysteine (+57.0214) and the addition of the TMT 6plex mass tag (+229.163) to peptide N-termini and lysine as fixed modifications. Searches were performed with full tryptic digestion and a maximum of 1 missed cleavage was allowed. The reverse database search option was enabled and all peptide data was filtered to satisfy false discovery rate (FDR) of 1%. Quantitation was done using a peak integration window tolerance of

0.0075Da with the integration method set as the most confident centroid. The resultant data file was filtered to remove proteins identified by less than 2 peptides. For each protein, the relative amount in the case compared to the amount in the control was presented as a ratio. The ratios were normalized by adjusting the values relative to the median ratio value which was set at 1. A full list of the proteins and associated details is shown in Supplementary Table 1.

Bioinformatics

Initially a bottom-up (shotgun) approach was used to interrogate the proteomics data, in particular candidate proteins whose deficiencies have previously been described to cause RBC membrane defects resulting in altered osmotic fragility associated with spherocytosis were interrogated[19-21] (Table 2, results considered likely to be impactful in bold). Analysis revealed a greater than 2 fold decrease in the amounts of alpha and beta adducin in the case compared to the control and band 3 protein was found to decrease by 1.88 fold in the affected dog. Identifying multiple protein deficiencies is not uncommon in proteomic analysis of putative RBC membrane disorders and can often be explained by the presence of a molecular defect resulting in a primary deficiency with secondary deficiencies due to decreases in proteins with which the primary protein interacts. Studies have hypothesized that the type and amount of secondary protein deficiencies may be involved in the hematological and clinical outcome as a result of enhanced membrane destabilisation.[20,22,23] A concomitant decrease in band 3, alpha and beta adducin with preserved gamma adducin is the characteristic combination of protein deficiencies seen with a primary band 3 protein deficiency, with evidence that the band 3-adducin bridge is crucial to membrane stability.[24] Primary band 3 deficiency also results in variable decreases in protein 4.2, which may also account for the identified decrease in this protein level.[23]

Proteins that were found to be increased or decreased by ≥ 2 fold in the RBC protein preparation from the affected case compared to the control dog were analyzed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) v 6.7[25] and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) 9.1 database[26] to identify classes of proteins belonging to specific processes and pathways that were over-represented. A total of 408 proteins were identified with high confidence (identified by at least 2 peptide matches), of which 252 proteins were found to be increased or decreased more than two fold in the patient compared to the control (Supplementary data – S1). In this study a two-fold change was taken as the cut-off value to identify proteins that were significantly altered in amount[7]. This approach allowed exploration of the protein differences identified in the presence of a reticulocytosis and hemolysis as previously described[5,27-30]. For proteins that increased in amount in the affected dog compared to the control, DAVID analysis revealed an enrichment of proteins associated with the functional annotation terms "citrate cycle (TCA cycle)"; and "oxidative phosphorylation" whilst proteins that decreased were associated with the functional annotation terms "proteasome"; "chaperonin-containing T-complex" and "intermediate filament protein" (Table 3). These results were supported by STRING network analysis that revealed a significant enrichment of proteins that increased/decreased in amount in known interaction networks that were associated with the KEGG pathway terms "metabolic pathways" and "proteasome" respectively (Figures 4 and 5).

Analysis of proteins in specific functional annotation clusters revealed that proteins previously described as up or down regulated in the presence of a reticulocytosis were altered in the affected dog compared to the control (see Table 4) [27,28,30]. Results were in agreement with previous studies and revealed the affected dog had decreased amounts of carbonic

anhydrase 1 and 2 and increases in proteins associated with organelles including calregulin, the 60-kDa heat shock protein, ATPase synthase subunit alpha and the 78-kDa glucose regulatory protein compared to the control dog. In addition, there were increased amounts of CD55 (decay accelerating factor), transferrin receptor, erythrocyte band 7 and prohibitin in the case compared to the control, these proteins are known to be unique to reticulocytes. Carbohydrate metabolism was also altered as previously described, including decreased malate dehydrogenase[28].

This study also uncovered protein alterations previously identified in studies of people with hereditary hemolytic anemia, hypothesized to be related to the presence of increased red blood cell turnover and increased oxidative injury (see Table 5) [5,29]. In particular, the affected dog displayed alterations in proteins involved in the cytoskeleton (e.g. GTPases), oxidative stress and endogenous protein quality control pathways (e.g. those involved in the glutathione synthetic pathway, chaperones and heat shock proteins) and down-regulated proteins involved in the ubiquitin-proteasome system including T complex proteins and 26S proteasome subunits.

The dog was managed with oral folate and is alive at 4 years following diagnosis. Follow-up hematologies have revealed stable results. The dog reportedly displays occasional reluctance to exercise, particularly the day after vigorous play, but is otherwise free of clinical signs.

Discussion

Proteomics was applied to further investigate presumed hereditary hemolytic anemia in a dog. The results suggested alterations in amounts of key membrane proteins in particular band 3 and alpha and beta adducin whilst other protein levels were comparable to the control

dog including alpha and beta spectrin and ankyrin. Interestingly, this same pattern of protein derangements has previously been reported in people with a diagnosis of a primary band 3 deficiency[29]. The proteomic approach used here is recognized as a diagnostic approach when investigating hereditary spherocytosis in people[5-7] however, to the authors' knowledge this technique has not been previously applied to a dog. Proteomics also uncovered a difference in protein levels between the affected and control dog, highly consistent with previous descriptions accounted for by the presence of a reticulocytosis and ongoing haemolysis[27,28,30].

A primary band 3 protein deficiency is the most common membrane defect described in people with hereditary spherocytosis with South European ancestry, and is typically inherited in a dominant fashion, with *de novo* mutations that are described. The severity of clinical signs appear to be associated with the extent of the deficiency and individuals often present in adulthood.[31]

Red blood cell membrane protein defects resulting in increased OF have been described previously in dogs. Spectrin deficiency was identified using a radioimmune assay (RIA) in a population of Dutch Golden Retrievers with persistent increased OF[3]. In addition, protein band 4.1 deficiency was identified in a dog with compensated hemolysis and persistent reticulocytosis using SDS-PAGE.[32] In contrast, two recent studies in cats described a strong suspicion of a RBC membrane defect but were unable to elucidate the protein responsible[1,33] despite one study using SDS-PAGE. SDS-PAGE analysis of the RBC proteome was not performed for the affected dog and may have uncovered protein alterations without the requirement for mass spectrometry (either in conjunction with SDS-PAGE or as part of quantitative proteomics). Given the improved sensitivity of a quantitative bottom-up proteomic approach[21] over SDS-PAGE (in the absence of concurrent mass spectrometry)

there may be a role for this as a tool to screen for candidate proteins in such cases with unexplained osmotic fragility in the future. Recently, next-generation sequencing has been used to identify genetic mutations that result in RBC membrane deficiencies [34]. Next-generation sequencing is complementary to the approach used in this investigation and its use in this investigation could have further supported the hypothesis regarding the affected case generated by proteomic analysis.[34]

In people with hereditary spherocytosis consensus recommendations for treatment are described[6]. Splenectomy is advised for severely or moderately affected individuals as the spleen is thought to be a hostile environment for the fragile RBCs (due to its low pH and oxidative stress) and folate supplementation was previously implemented for all cases, although now recommendations reserve it for circumstances where a folate deficiency is possible (e.g. socioeconomic environment such as poverty) or in moderate and severe cases. The justification for supplementation in the affected dog was that despite normal serum folate levels, a predominantly fish-based diet meant it was considered at mild risk of low dietary intake. Approximately 15% of people are asymptomatic apart from during periods of erythroid stress such as viral illness or splenomegaly therefore urgent medical attention is advised in the event of any febrile illness and antimicrobial prophylaxis recommended if any surgery is performed given the risk of decompensation.

Interestingly, many of the proteins up and down regulated in human patients with hemolytic anemia and reticulocytosis were also identified as differentially expressed in the affected dog compared to the control dog. Previous studies have described techniques to reduce reticulocyte numbers prior to analysis,[35] whilst extensive RBC washing and elimination of the top layer of the RBC pellet after centrifugation was performed specific attempts to reduce

reticulocyte numbers were not performed in this study, although this is unlikely to have had a significant impact upon the analysis given the relatively small proportion of reticulocytes in the sample.

A limitation of the study was the numbers of samples analyzed, in similar studies of human patients with hereditary spherocytosis small numbers of control subjects are also described with an emphasis on the quantitative alterations in proteins in these patients[5,29]. In spite of this limitation the comparable findings to studies exploring the RBC membrane proteome in human patients with hereditary spherocytosis imply relevant findings, although further studies into the canine RBC proteome would be valuable in supporting this result.

In conclusion, high-throughput proteomic analysis enabled detailed exploration of the protein changes associated with non-immune mediated hemolytic anemia in a dog. Interestingly, identified protein alterations displayed strong similarities to those described in people investigated for hereditary spherocytosis using proteomics.

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Table 1 - Serial hematology results

Parameter	Day 1	Day 7	Day 30	Day 31	Day 61	Day 150	Day 420	Day 1095	Reference range
Hb (g/dl)	13.1	15.6	15.0	12.5	14.5	14.3	15.8	14.6	12.0-18.0
HCT (L/L)	43.0	49.0	51.0	41.8	49.5	47.4	54.2	45.0	35.0-55.0
RBC (x 10^9/l)	5.94	6.93	6.80	5.58	6.61	6.33	7.33	6.89	5.00-8.50
MCV (fl)	72.4	70.7	75.0	74.8	74.9	74.9	73.9	65.3	65.0-75.0
MCHC (g/dl)	**30.5**	**31.8**	**29.4**	**29.8**	**29.3**	**30.1**	**29.1**	**32.4**	34.0-37.0
Reticulocytes (x 10^{12}/l)	**229.9**	**478**	**310**	**370**	**570**	**670**	**350**	**352**	< 60
Blood smear evaluation	**Spherocytes (1+)**	**Spherocytes (1+)**	**Anisocytosis (1+)**	**Spherocytes (1+)**	**Anisocytosis (1+)**	**Anisocytosis (1+)**	**Anisocytosis (1+)**	**Anisocytosis (1+)**	-

Table 2 – Proteomic results for candidate proteins

Candidate protein	**Uniprot acc.no.**	**MW (kDa)**	**pI**	**% Coverage**	**Protein Ratio (case/control)**
Ankyrin-1	F1PRD8	203.8	6.19	25.96	0.732
Spectrin alpha chain	E2R1P6	118.0	4.89	30.23	0.834
Spectrin beta chain	F1Q253	268.0	5.31	28.80	0.828
Protein 4.1	J9P7E1	93.2	5.64	28.40	0.880
Protein 4.2	F1PD51	77.4	6.79	20.78	0.640
Anion exchange protein 3 (Band 3)	**J9NWP9**	**95.1**	**6.57**	**17.02**	**0.543**
Alpha Adducin	**E2RJW7**	**81.1**	**5.76**	**20.49**	**0.475**
Beta Adducin	**F6XYK7**	**79.6**	**5.92**	**29.36**	**0.359**
Gamma Adducin	E2QZE7	79.0	6.18	3.82	1.255

Table 3. DAVID analysis of proteins significantly altered in amount in RBC membranes isolated from case compared to the control dog.

	Functional annotation term[a]
>2 fold increase	Citrate cycle (TCA cycle) (10.37)
	Fatty acid beta-oxidation (8.12)
	Protein folding/cell redox homeostasis/oxidoreductase (5.18)
	Oxidative phosphorylation (5.1)
>2 fold decrease	Proteasome (7.84)
	Chaperonin-containing T-complex (6.18)
	Intermediate filament (5)
	Proteasome regulatory particle, base subcomplex (3.93)

[a]The total list of proteins that showed a >2 fold change in abundance in RBC membranes from the case compared to the control dog were analyzed for the presence of enriched groups of proteins and proteins involved in specific host pathways using DAVID. The functional descriptors for the 4 clusters of proteins that were most enriched for each category (with an EASE score greater than 1.3 (corresponding to a *p* value < 0.05)) are shown with the corresponding EASE score.

Table 4 – Protein changes previously accounted for by presence of reticulocytosis

Protein	Uniprot acc.no.	MW (kDa)	pI	% Coverage	Protein Ratio (case/control)
Carbonic anhydrase 1	F1PBK6	29.0	7.12	11.49	0.323
Carbonic anhydrase 2	F1PDY8	29.2	7.18	26.92	0.264
Calregulin	F6UYJ9	47.1	4.55	4.19	2.677
60 kDa Heat Shock Protein	E2QUU5	61.0	6.00	19.72	7.916
ATP synthase subunit alpha	E2RNG2	59.6	9.04	26.58	5.904
78kDa glucose regulatory protein	F1PIC7	72.2	5.16	21.71	2.404
C55 (decay accelerating factor)	F1PCI2	37.1	7.78	13.06	2.530
Transferrin receptor	F1PEN6	86.5	6.30	7.66	3.732
Erythrocyte band 7	F1PFS1	25.6	7.11	24.46	2.632
Prohibitin	A8W340	29.8	5.76	28.68	6.286
Malate dehydrogenase	Q0QF34	31.1	7.64	20.54	4.443

Table 5 – Protein changes previously accounted for by hemolysis

Protein	Uniprot acc.no.	MW (kDa)	pI	% Coverage	Protein Ratio (case/control)
CDC42 (GTPase)	P60952	21.2	6.55	16.75	0.348
Glutathione peroxidase	F6XH46	17.3	5.57	25.32	0.434
14-3-3 protein epsilon (Chaperone)	J9P9V0	14.4	5.12	12.9	0.113
Heat shock 70kDa protein	E2RT63	94.3	5.16	13.93	0.193
CCT8 (T complex protein)	E2RQ81	59.7	5.68	10.04	0.327
PSMC2 (26S proteasome subunit)	F1PPH7	48.6	5.95	19.63	0.269

Figure 1 – Hemoglobin-oxygen dissociation curve

A graph depicting haemoglobin-oxygen dissociation curve from the case and a healthy age-matched control dog. The percentage saturation is shown on the vertical axis and the increments of partial pressure oxygen on the horizontal axis. The one-half saturation point (p50) is found to be comparable between the case and control dog (case p50 28.3mmHg, reference range 26-30mmHg, control 29.1mmHg).

Figure 2 – Osmotic fragility test

Osmotic fragility (OF) test results from the case and healthy age-match control dog. The curve for the affected case is right-shifted consistent with increased OF and hemolysis occurring at higher concentrations of sodium chloride.

Figure 3 - Electron microscopy of case RBCs

Scanning electron microscopic pictures of red blood cells (RBCs) from the affected dog, no obvious abnormalities were identified.

Figure 4 and 5. Protein association networks detected using the STRING database.

Proteins that were altered in abundance either figure 4) > 2 or figure 5) < 2 fold respectively in the RBC membranes from the affected dog compared to the control dog were analyzed using STRING. The nodes shaded in red indicate proteins that were most significantly enriched for a specific GO Biological Process/KEGG pathway term in each STRING network displayed. For the proteins that increased >2 fold the KEGG pathway term "Metabolic pathways" ($P = 3.72 \times 10^{-25}$, gene set = 49)" was identified, whilst for proteins that decreased >2 fold, the GO/KEGG pathway term "Proteasome" ($P = 1.06 \times 10^{-32}$, gene set = 20) was identified.

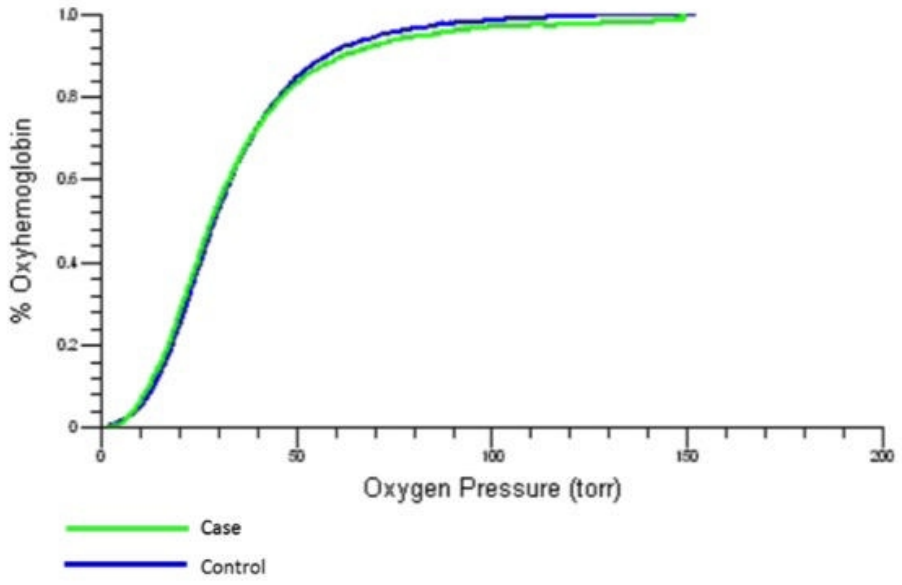

Figure 1

289x159mm (72 x 72 DPI)

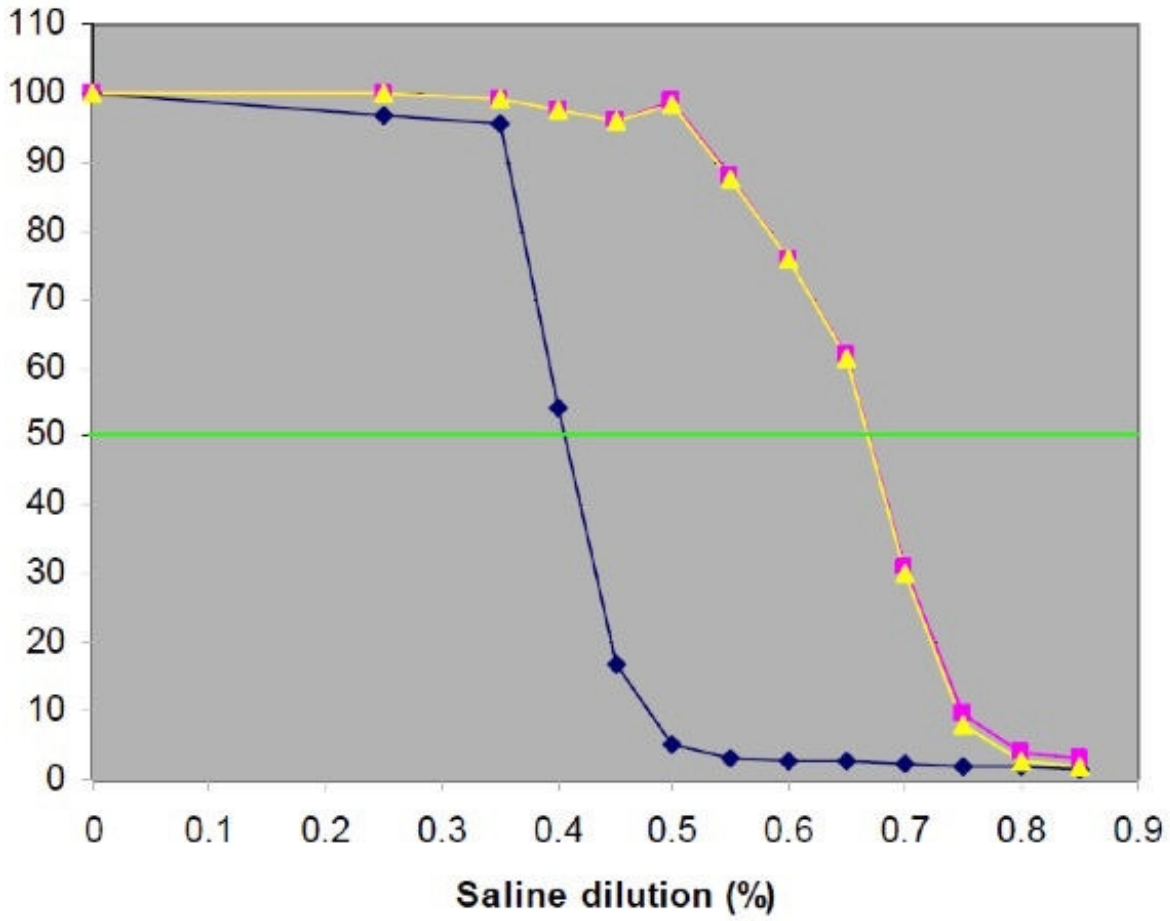

Figure 2

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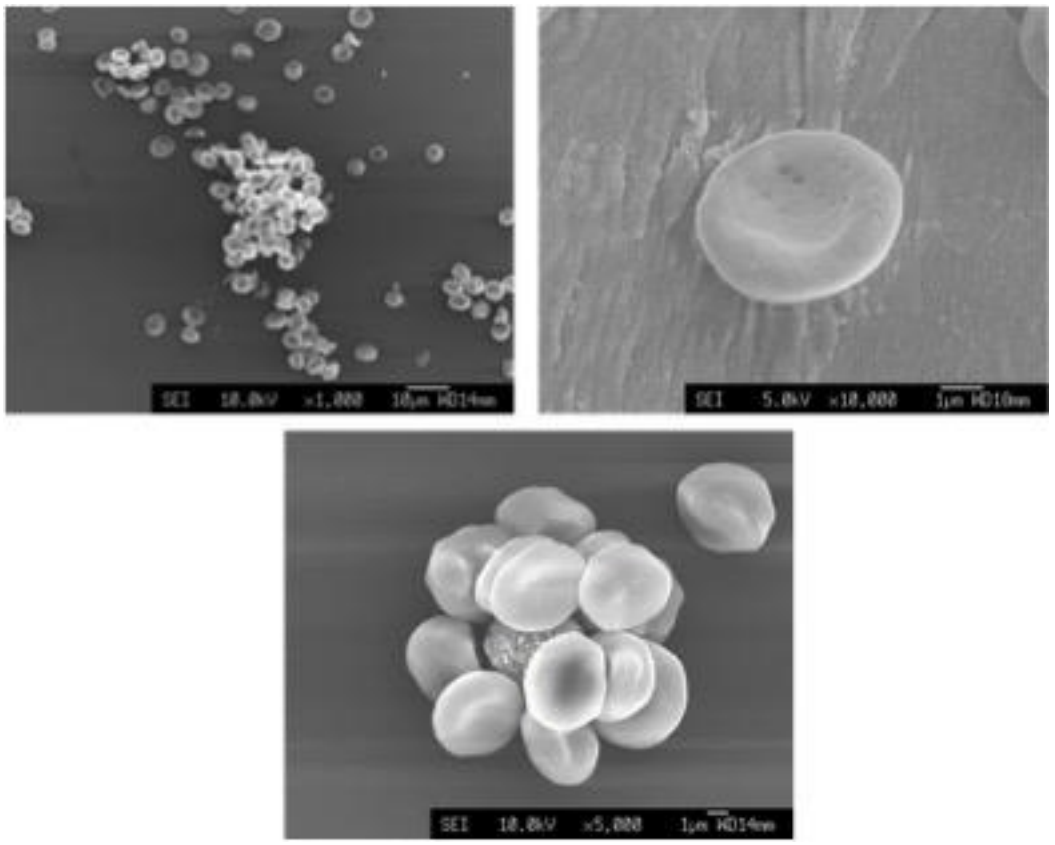

Figure 3

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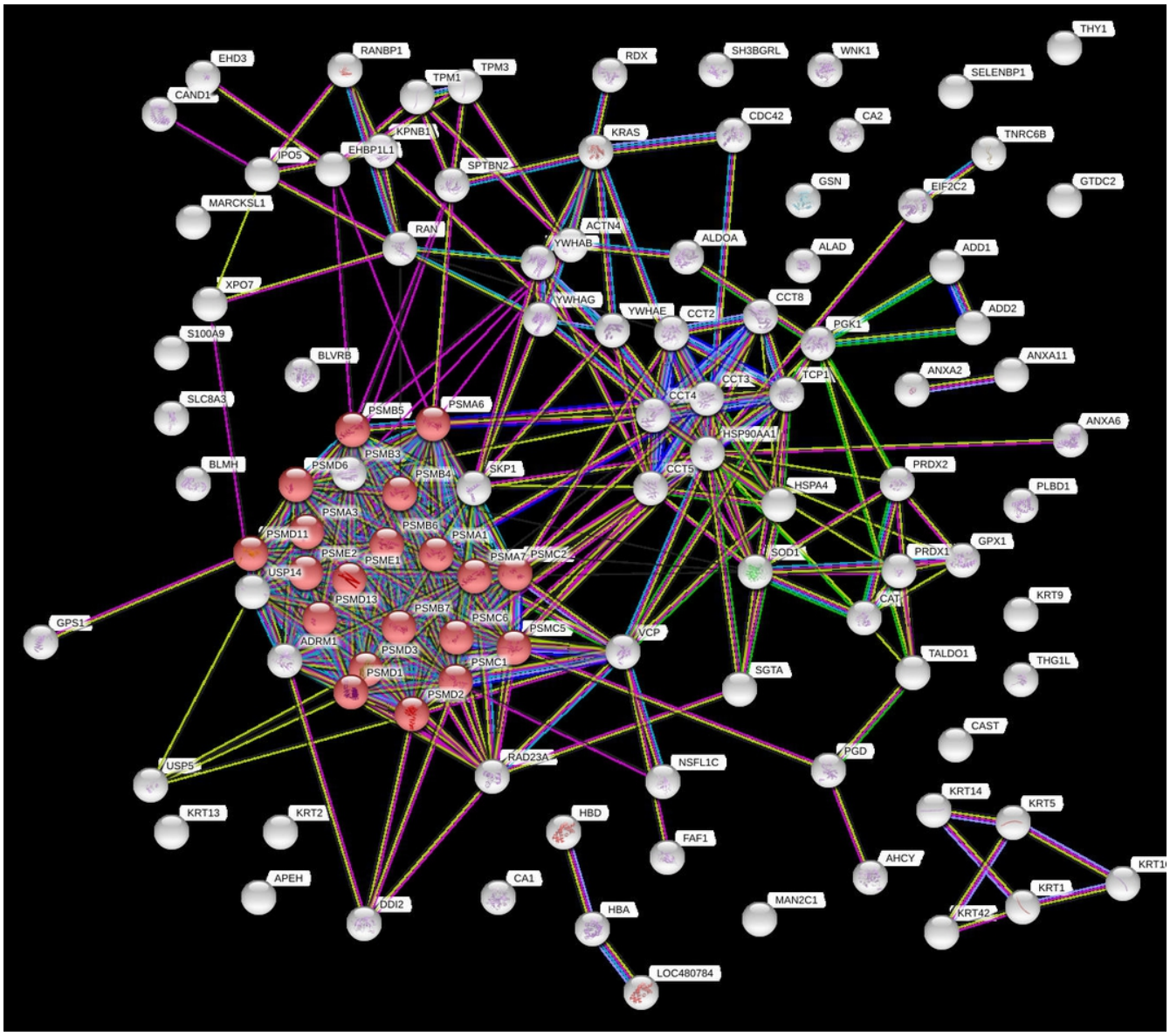

Figure 4

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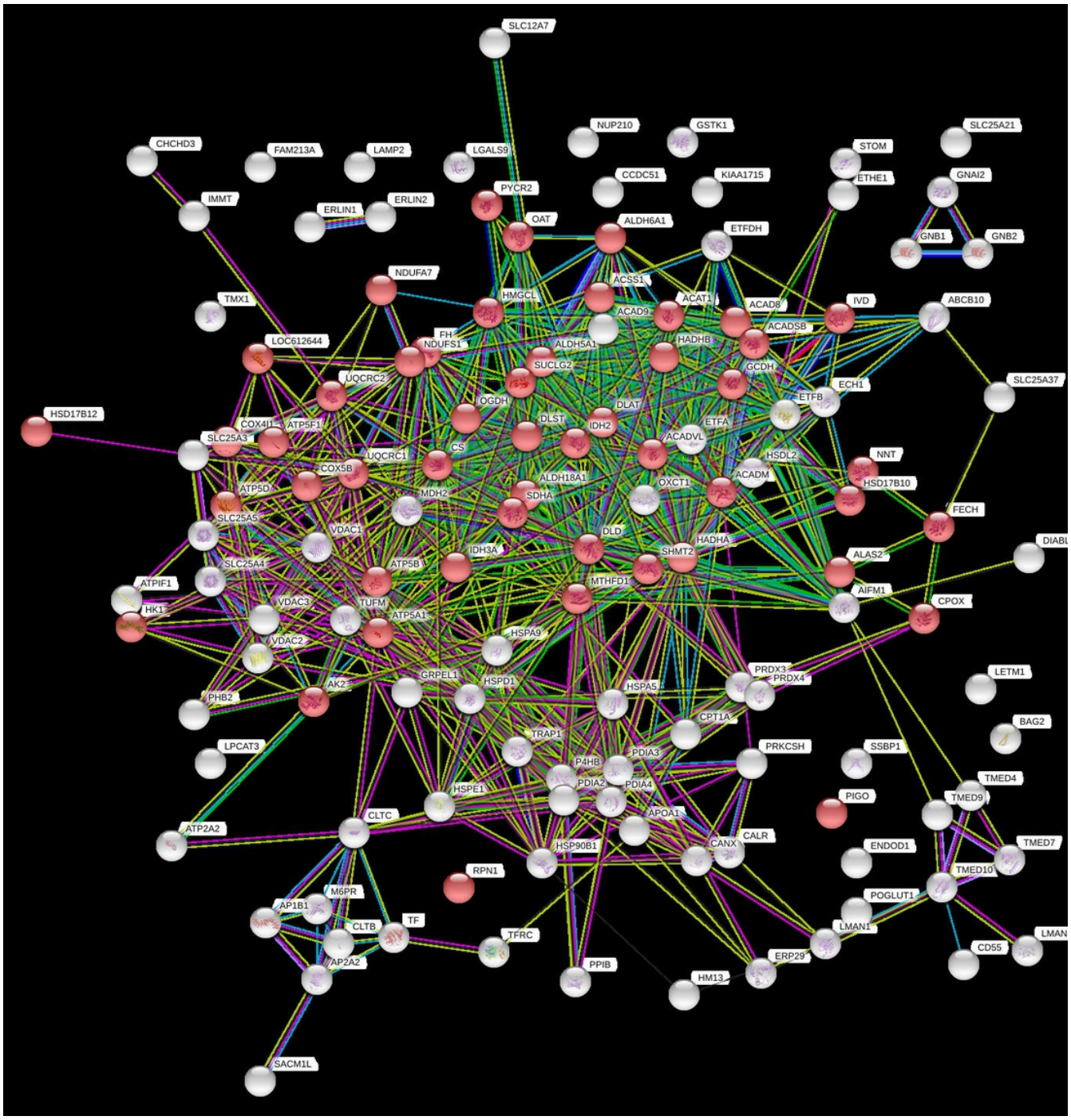

Figure 5

386x403mm (72 x 72 DPI)